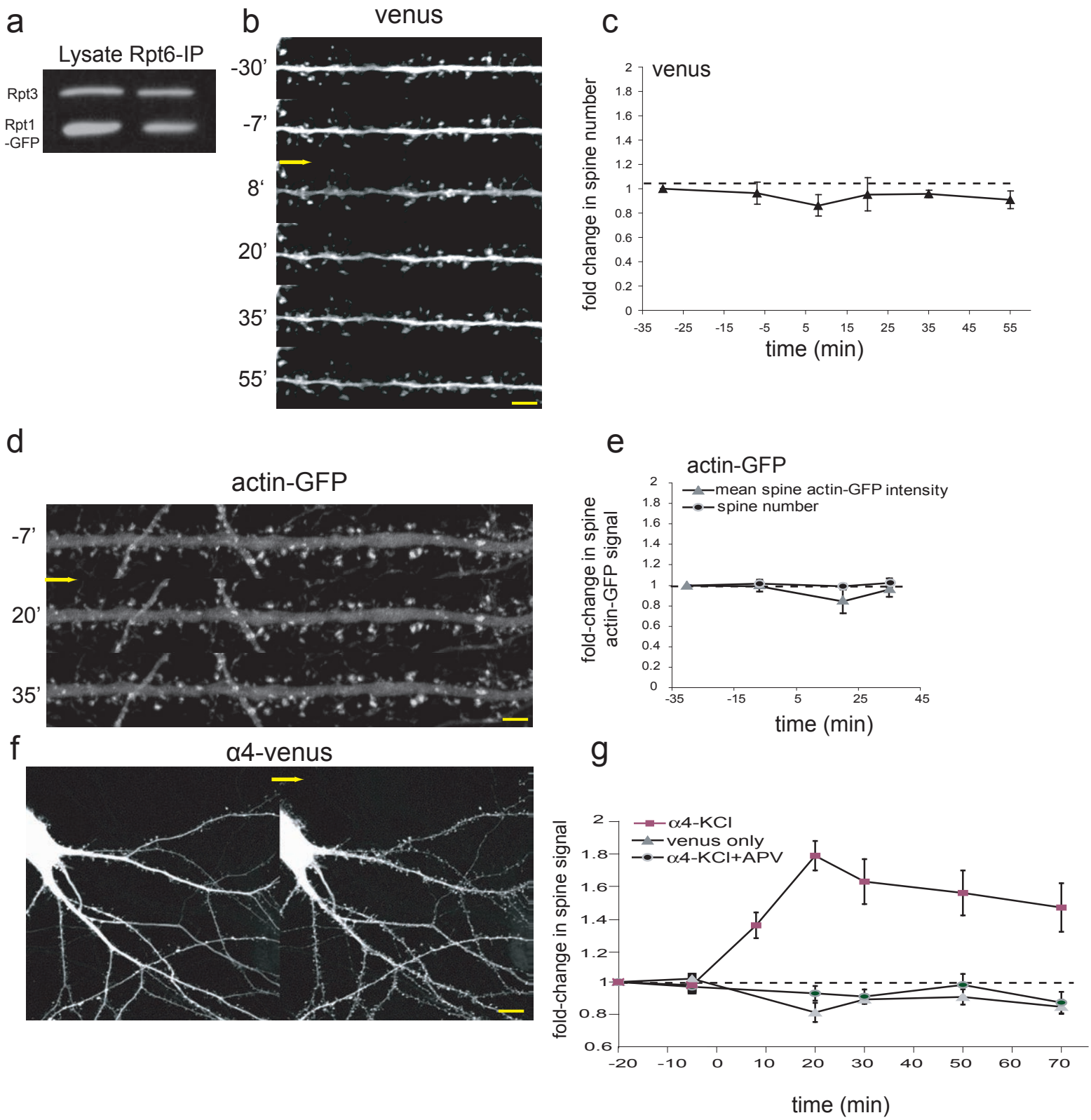
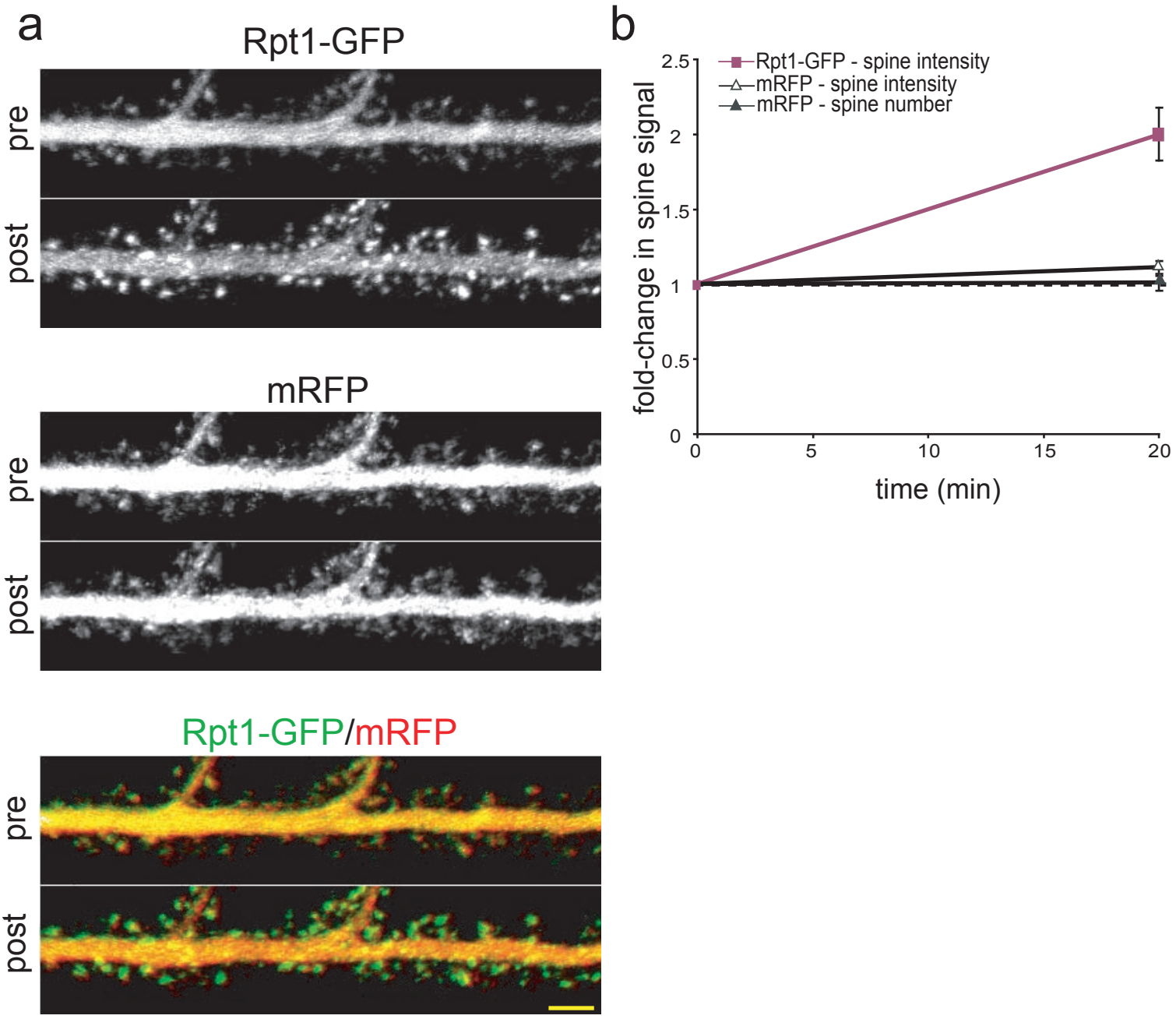
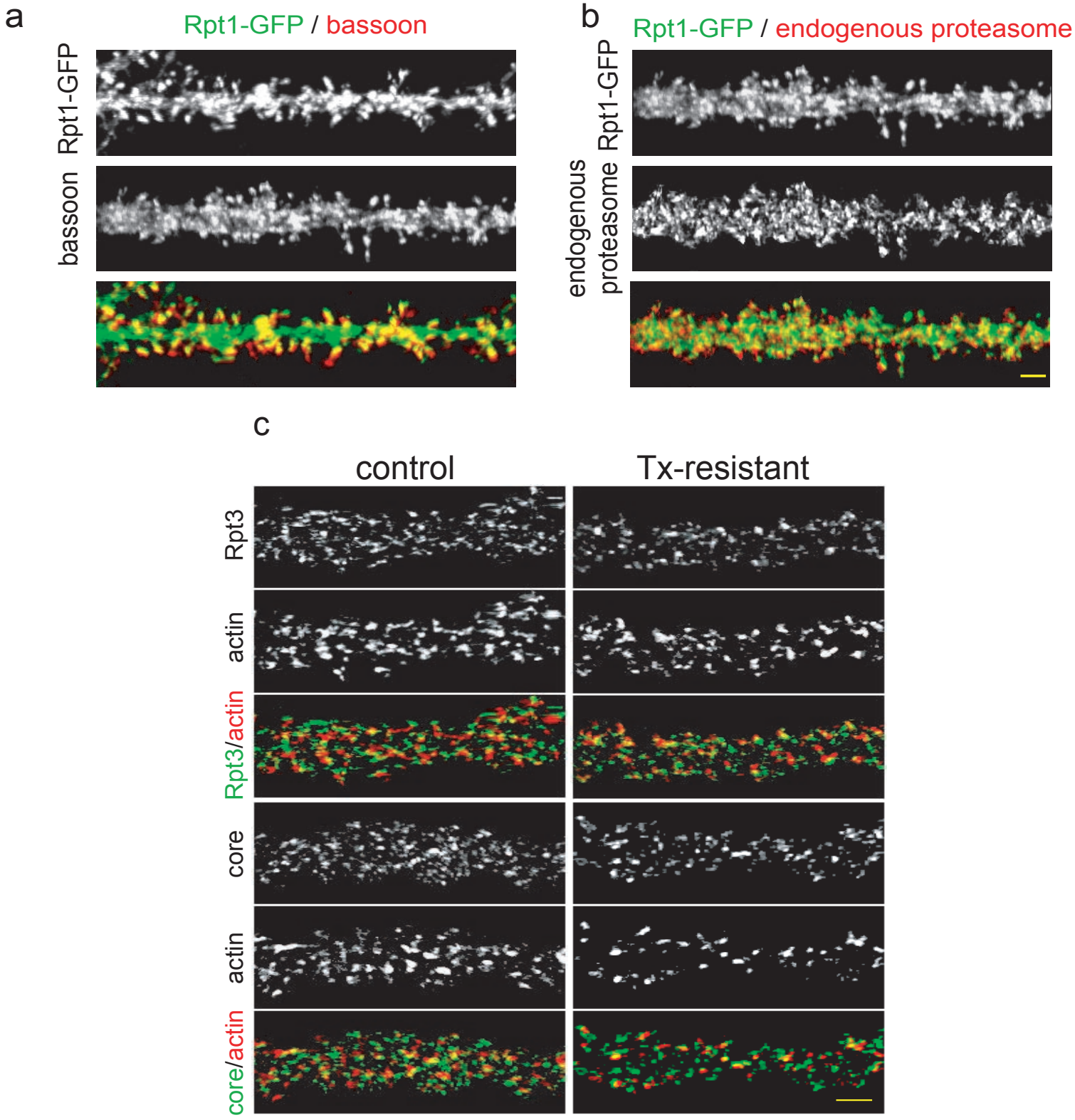


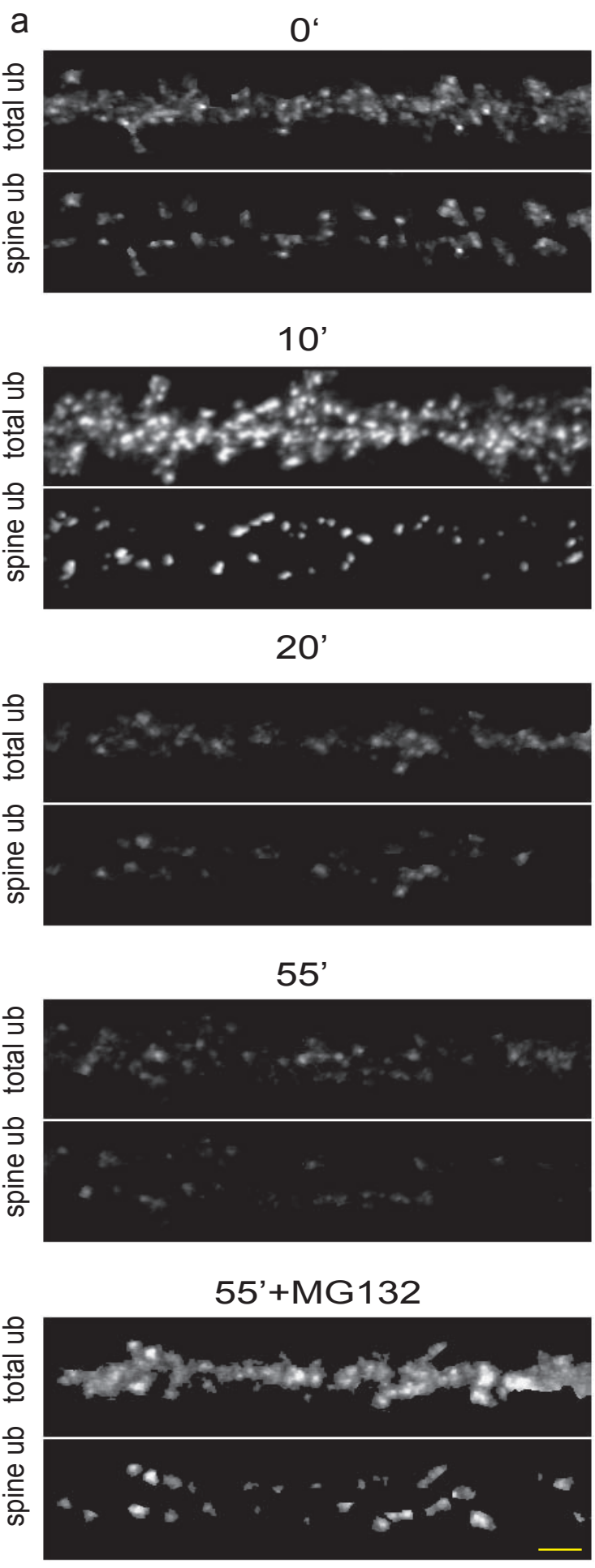
Supplementary Fig. 1. a, The Rpt1-GFP subunit is incorporated into the proteasome with similar efficiency as an endogenous proteasome subunit. Comparison of the lysate:IP ratio on the western blot indicates that the incorporation efficiency of Rpt1-GFP is 77.4 ± 3.4% (from 2 independent experiments) of the endogenous proteasome subunit Rpt3. (lysate:IP ratio is 1:1.) b, Spine number does not change with KCl stimulation (arrow). Scale bar = 4.5 μm. c, analysis of spine number before and after KCl stimulation (n = 20 cells from 3 independent experiments). d, Actin-GFP does not show redistribution into spines with KCl stimulation (arrow). Scale bar = 4 μm e, Analysis of actin-GFP signal before and after KCl stimulation for the mean spine intensity and spine number (n = 10 cells from 3 independent experiments). f, α4-venus moves into spines with KCl stimulation. Scale bar = 10 μm. g, Group data of α4-venus experiments. APV blocked the KCl-induced trafficking of α4 into spines and spine area does not change with KCl stimulation (n = 21 for each group from 4 independent experiments, $p<0.05$ by using ANOVA at t=8' and thereafter, error bars denote s.e.m.).

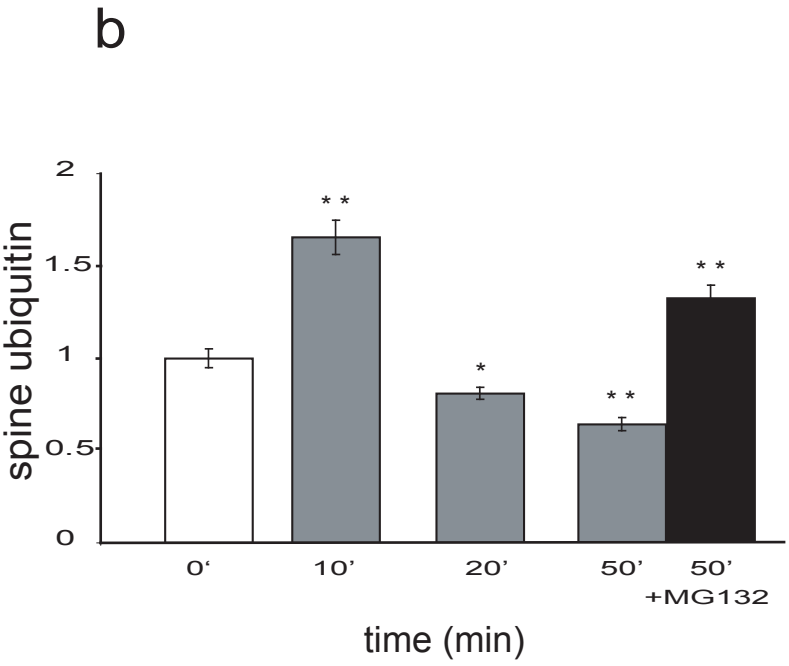


Supplementary Fig 2. In order to examine whether there are changes in spine number or volume following our stimulation, we expressed mRFP along with Rpt1-GFP in the same neurons using a Sindbis virus (Rpt1-GFP-IRES-mRFP). Rpt1-GFP moves into spines with no significant change in the spines of the same neuron. a, A dendritic segment expressing Rpt1-GFP and mRFP before and after KCl stimulation. A composite image is shown in the bottom panel. Scale bar = 3.8 µm. There is no change in absolute spine number before and after stimulation. b, Summary data (n=21 cells from 3 independent experiments. $p<0.05$ only for Rpt-1-GFP group by using Student's t-test, error bars denote s.e.m.). Although there was no significant change in the spine number or the spine mRFP signal intensity following KCl stimulation, there was still a ~90% increase in spine Rpt1-GFP signal in the same neurons (20 min post KCl).

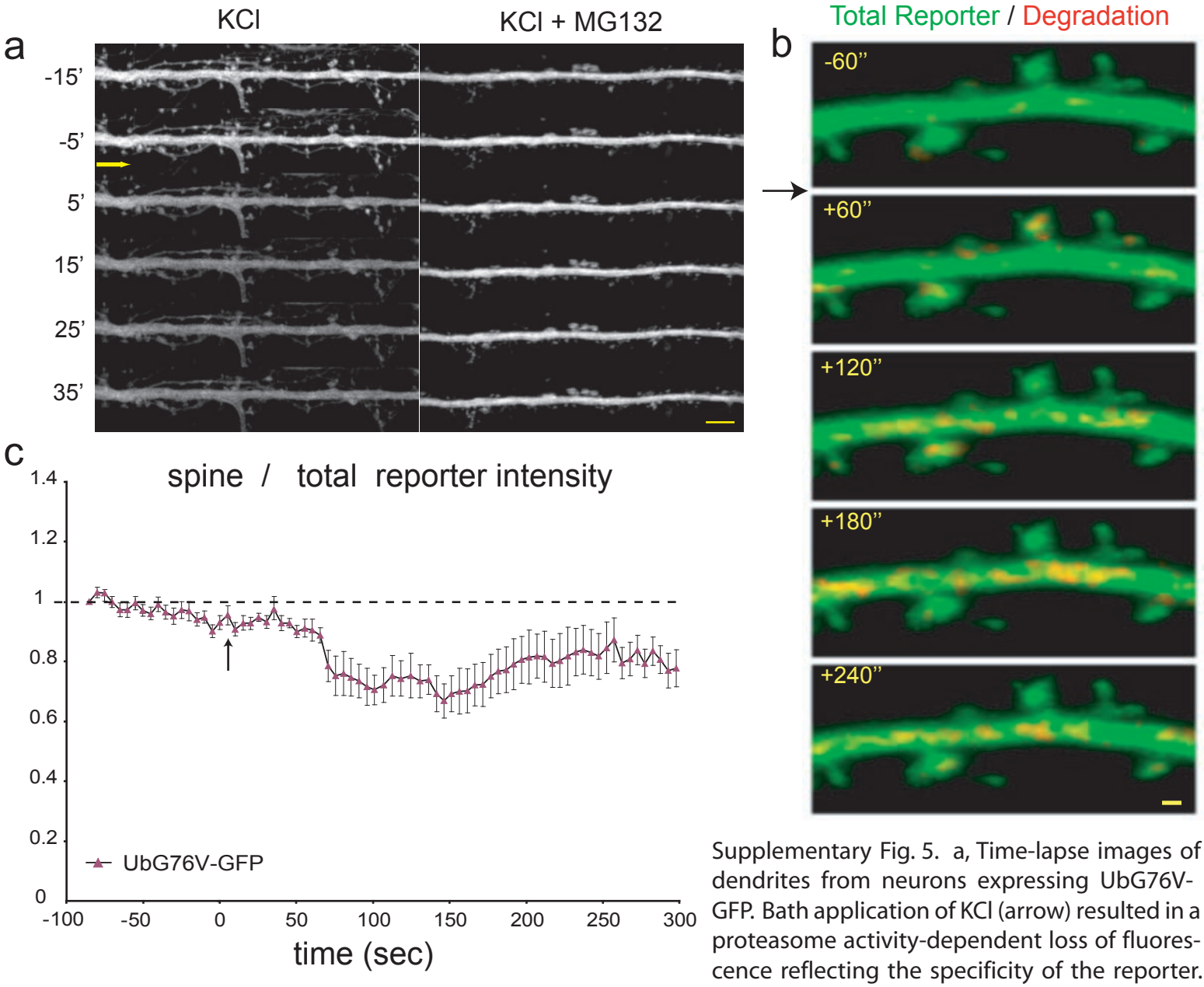


Supplementary Figure 3. a and b, Rpt1-GFP-inhabited spines are associated with presynaptic terminals and endogenous proteasome subunits. Representative images following stimulation. Retrospective immunostaining for the presynaptic marker bassoon (a) indicates that most spines are contacted by presynaptic terminals (Table S1) and (b) that Rpt1-GFP puncta are also immunopositive for endogenous core subunits of the proteasome (Table S1). Scale bar = 2.7 µm. c, Some proteasome colocalizes with the actin cytoskeleton in control and Tx-extracted neurons (Table S1). Scale bar = 3 µm.

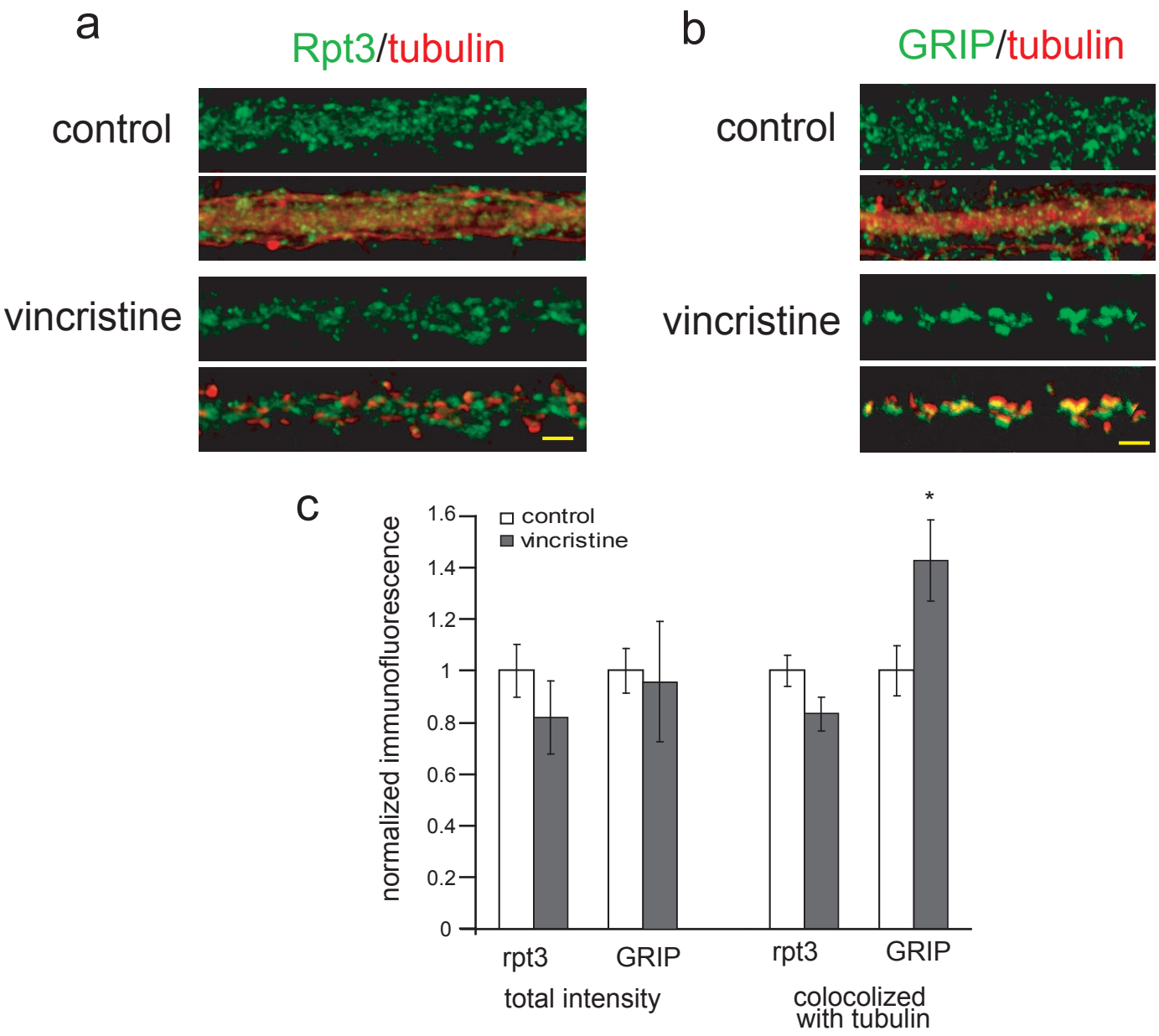




Supplementary Fig 4. a, Total and spine ubiquitin staining from control and KCl or KCl+MG132 stimulated neurons. Scale bar = 3.4 μm. b, Analysis of the ubiquitin staining shown in a (n=14 cells for each group from 3 experiments, $p < 0.05$ (*) and 0.01 (**) by using Student's t-test, error bars denote s.e.m.). The decrease in ubiquitination levels was blocked by the proteasome inhibitor MG132. These results suggest that stimulation causes an initial increase in ubiquitination possibly due to activation of the ubiquitination machinery. This increase is followed by the degradation of these ubiquitinated proteins by the proteasome.



Supplementary Fig. 5. a, Time-lapse images of dendrites from neurons expressing UbG76V-GFP. Bath application of KCl (arrow) resulted in a proteasome activity-dependent loss of fluorescence reflecting the specificity of the reporter. (see Fig 3b) Arrow indicates the start of the stimulation. Scale bar = 7.5 µm. b, Localization of the degradation to spines just after stimulation. Arrow indicates the start of the stimulation. green: total reporter signal at the beginning of the experiment. red: the difference between the image at the indicated time point and the very next image in the time series. Thus, red pixels represent the amount and localization of the reporter degradation at that point. Each difference image (red) is superimposed on the dendritic segment analyzed (green) in order to show the spatial localization of the degradation along the dendrite. Notice that the degradation occurs first at spines compared to the rest of the dendrite. This is followed by equal rates of reporter degradation in spines and the shaft possibly due to diffusion of the reporter from shaft to the spines. Another possibility is that the "residual" shaft proteasome is sufficient to degrade some the of the shaft reporter. Scale bar = 1.5 µm. c, Immediately after stimulation, the proteasome reporter, UbG76V-GFP, is degraded faster in spines when compared to the shaft. Arrow indicates the start of the stimulation. d and e, UbG76V-GFP expressing neurons (in green) and the position of the local perfusion (in red) shown in Fig 3c,d. (* : control perfusion, **: NMDA perfused dendrite, ***: non-perfused dendrite) Scale bar = 20 µm.



Supplementary Fig. 6 To test the role of the microtubule-based network, we examined whether microtubule depolymerization by vincristine[21] altered the distribution of the endogenous proteasome. After vincristine treatment, individual microtubules disappeared and tubulin paracrystals formed, characteristic of vincristine treatment[21,22] a, Microtubule depolymerization with vincristine has no significant effect on proteasome distribution. Furthermore, the temperature at which the detergent extractions were performed (4 °C) suggests that the microtubule-based cytoskeleton is not involved in the proteasome sequestration, as microtubules depolymerize at cold temperatures. Scale bar = 5 µm. b, Another neuronal protein, GRIP1, which is known to associate with microtubules in neurons[23], however, did exhibit clustering after vincristine treatment. Scale bar = 4.6 µm. c, Group analysis of the vincristine experiment shown in a and b. (n = 18 cells for each group from 3 independent experiments, $p<0.05$ for the increase in the colocalization between GRIP and tubulin after vincristine treatment).

Supplementary Methods:

Sindbis Virus Constructs. Rpt1-GFP, a tagged ATPase-type subunit of the regulatory 19S cap complex, (yeast) coding sequence was amplified from the pBS-Rpt1-GFPHA-HU plasmid[16] with PCR and subcloned in to pcDNA3.1 and subsequently to pSinRep5 vector (Invitrogen). To generate the α4-venus Sindbis virus, the α4 (MGC# 2581897) cDNA clone in pSPORT6 and venus clone in pCS2-venus[30] were used to amplify α4 and venus PCR products, respectively. Then, venus was cloned in to pcDNA3.1 (venus-pcDNA3.1), followed by the cloning of α4 in to the upstream of the venus coding sequence. The α4-venus was then subcloned into the pDNR-1 vector followed by recombination into pSinRep5-loxP acceptor vector using the cre recombinase (BD Biosciences). In order to test the incorporation efficiency of Rpt1-GFP in to the endogeneous proteasome structure, endogenous proteasome from cultures expressing Rpt1-GFP was immunoprecipitated with a subunit specific antibody against the Rpt6 subunit. The immunoprecipitates were analyzed for the Rpt1-GFP and another endogenous proteasome subunit, Rpt3. (Sup.Fig. 1a). The degradation reporter, Ub^{G76V}-GFP, developed by another group[19,20], consists of GFP carrying a constitutively active degradation signal. The Ub^{G76V}-GFP reporter possesses a ubiquitin fusion degradation signal consisting of an N-terminally linked ubiquitin that can serve as an acceptor for polyubiquitin trees. The G76V mutation prevents the removal of ubiquitin by cellular ubiquitin hydrolases, leading to the ubiquitination and proteasomal degradation of the entire Ub-GFP fusion. The Ub^{G76V}-GFP coding sequence was cloned into pSinRep5 from the EGFP-N1-Ub^{G76V}-GFP vector by standard cloning techniques. Rpt1-GFP-IRES-mRFP was generated from the pSinRep5-Rpt1-GFP clone by inserting the IRES (pLP-

IRES2-EGFP) and mRFP sequences downstream of Rpt1-GFP sequence. The Sindbis virus was produced according the instructions provided by the manufacturer (Invitrogen). Ambion SP6 mMessage Kit was used to produce the RNA for virus production. GFP and YFP-actin Sindbis viruses were described earlier[31] .

Cultured hippocampal neurons. Dissociated postnatal (P1-2) rat hippocampal neuron cultures were prepared as previously described[32], and plated at a density of 230–460 mm^2. Neurons cultured for 18-21 DIV were used in all experiments. Neurons were infected by washing them twice with HEPES-buffered solution (HBS) (110 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM D-glucose, and 10 mM HEPES-NaOH (pH 7.4)) (37° C), followed by 30 min incubation with the diluted Sindbis virus in conditioned growth media. After infection, cells were washed twice with HBS (37° C) and were further incubated for 4 hours (for Ub^{G76V}-GFP) or 12 hours (for GFP- and Venus-tagged proteasome subunit expression). The shorter expression period for Ub^{G76V}-GFP was essential to get a high signal-to-noise ratio for the proteasome activity detection.

Live imaging of cultured neurons. *Bath stimulation of neurons.* To isolate the dynamics of existing proteasome subunits, neurons were kept in anisomycin prior to and during the live imaging of UbG76V and GFP-tagged proteasome subunits. During the experiment, cells were continuously bath perfused with HBS/aniso (flow rate =1.5 ml/min) at 37° C. Cells were stimulated with either KCl (1.5 minutes, high KCl-HBS (same as HBS except for 55 mM NaCl and 60 mM KCl)) or NMDA (20 μM, 3 min) by switching the perfusion medium. APV (50 μM, 1h) and MG132 (10 μM, 10 min) were used to antagonize NMDA receptors and proteasome activity, respectively. The cells were imaged with a Zeiss LSM 510 microscope with 40X oil objective and 2X zoom. Z-

stacks were acquired at the indicated time points and flattened. In order to quantify the spine fluorescent signal, all the images in the time series were summed and all the "protrusions" away from the dendritic surface were selected as spines on the summed image. Then the percent change in the mean intensity of the spine signal over time is quantified. For the venus, actin-GFP and mRFP controls, the mean area of spine and the mean number of spines per dendritic length were also calculated. Analysis of spine signals were conducted by a reviewer blind to the experimental condition. *Local stimulation.* The local perfusion set-up was as previously described[33]. Before perfusion, the neurons were kept in HBS (37° C) for 1 hour with 40 μM anisomycin to block protein synthesis. In order to visualize the local protein degradation, dendritic segments of cultured hippocampal neurons expressing the Ub^{G76V}-GFP proteasome activity reporter were perfused locally with 50 μM NMDA for 6 minutes. The perfusion pipette contained Alexa-568 dye in order to visualize and quantify the dimensions of the perfusion spot. Cells were also bath perfused with HBS (37° C) during local stimulation. Images were acquired on Olympus IX50 microscope with 40X air objective lens and 2X zoom using FluoView Image acquisition Software. Z-stacks were acquired every 2 minutes (2 baselines + 4 NMDA) with 0.5 μM slice thickness. After the images were flattened, the mean intensity of the GFP signal in the perfusion spot was compared over time and to the adjacent dendritic segments and non-perfused dendrites.

FRAP/FLIP. All of the FRAP/FLIP experiments were performed at 37°C with a Zeiss LSM 510 using a 40X oil objective and 4X zoom. The cultured neurons expressing Rpt1-GFP were imaged before stimulation and 3-4 spines with a long neck were chosen to be monitored. For the FRAP experiments, single z-slice images were taken in order to be

able to capture the bleaching of the Rpt1-GFP. After taking 5 baseline images, each spine was bleached one time for 150 msec using a laser intensity 200 times greater than that used for image acquisition. After bleaching, recovery of the fluorescence was measured as a function of time. For the FLIP experiments, after taking 3 baseline images, ~ 10 μm of the dendritic shaft was bleached repeatedly for 45 msec before taking the next image in the time series. The loss of the fluorescence from the spines adjacent to the bleached shaft region was monitored with time. After acquiring the pre stimulation data as described above, neurons were stimulated with KCl, and, and a second round of bleaching (same parameters) was performed on the same spines for the FRAP and the same dendritic shaft segment for the FLIP experiments.

The FRAP data were fit to a one phase exponential function by using the (Bottom to (Span +Bottom) analysis. ($Y= Span*(1 - \exp(-X/\tau)) + Bottom$) in the GraphPad Prism 4.3 software. Similarly, the FLIP data were fit to a one phase exponential decay function ($Y=Span*\exp(-X/\tau) + Plateau$) for the prestimulation curve. For the FLIP poststimulation experiments, the data was better fit by a linear function. The time constant (τ) and the immobile fraction were calculated from the exponential function equations.

Immunocytochemistry. Cultured neurons were processed for immunocytochemistry as previously described[32]. The antibodies that were used for immunocytochemistry were as follows: Chicken anti-GFP (Aves Labs), mouse anti-actinin-2 (clone EA-53, Sigma), mouse anti-bassoon (StressGen), rabbit core-proteasome (α5, α7, β1, β5i, and β7) (Calbiochem), rabbit core-proteasome (BIOMOL), rabbit anti-Rpt3 (BIOMOL), mouse anti-ubiquitin (FK2 clone, Affiniti), rabbit anti-GRIP CT (Upstate). The actin

cytoskeleton was stained with Rhodamine-Phalloidin (Molecular Probes). The secondary antibodies that were used for immunocytochemistry were as follows: Alexa 488 anti-Chicken, Alexa 647 anti-mouse, Alexa 488 anti-Rabbit, Alexa 546 anti-Mouse (all of the secondary antibodies were from Molecular Probes). All the image acquisition was performed with Zeiss LSM 510 Microscope with 40X oil objective and 2-3X zoom. To analyze immunocytochemistry images, LSM files were processed by NIH ImageJ and custom macros were used to quantify colocalization. All of the channels were processed for thresholding and watershed segmentation in 3D, and the total and colocalizing particles were quantified for the mean number, area, and the intensity. For the cytoskeleton experiments, Latrunculin A (5 μM, 24 h, Molecular Probes) and vincristine (5 μM, 5 h, Sigma) were used to disrupt the microfilaments and the microtubules, respectively[21]. For the endogenous proteasome experiments, cultured neurons were stimulated with KCl (1.5-3 mins, 60 mM) in conditioned medium and further incubated at 37°C for 12 min. After stimulation, the neurons were transferred quickly on to ice and processed for immunocytochemistry. For the FK2 immunostaining experiments, hippocampal cultures were infected with a Sindbis virus expressing GFP. The staining was performed for both the GFP and the ubiquitin and the GFP channel was used as a marker for the dendritic morphology. A reviewer blind to the FK2 signal outlined the "protrusions" from the dendritic surface using GFP template image. A spine mask was then created from these protrusions. The polyubiquitin FK2 signal overlapping with the spine mask was used for quantification. MG132 was added 5 minutes before stimulation for the 50 min. time point. Detergent extraction experiments were performed with 0.5 % TritonX-100 as described earlier[21]. Cultured neurons were stimulated with 20 μM

NMDA for 1 minute followed by immediate 50 μM APV wash in order to decrease the neurotoxic effects of NMDA. After NMDA stimulation cultures were incubated for another 19 minutes in the presence of APV. Control dishes received the APV incubation as well, but not the NMDA stimulation. After this stimulation protocol, dishes were processed for fixation with or without prior detergent extraction.

Slice stimulation, synaptosome preparation, and Western blot analysis. Hippocampal slices were prepared as described earlier[34]. Before stimulation, slices were submerged in a chamber and continuously perfused with 32°C artificial cerebral spinal fluid (119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 11.0 mM D-glucose). Stimulation was performed with ACSF containing 60 mM KCl for 6 minutes. 30 minutes after KCl stimulation, slices were immediately transferred into cold ACSF and processed for synaptosome preparation as described earlier[35]. Synaptosomes were lysed in 0.2% SDS followed by 1% TritonX-100 containing lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, protease inhibitor mix (Roche)) and equal amounts of proteins were resolved on 10% SDS-PAGE. The antibodies used for Western blotting analysis were as follows: chicken anti-GFP (Aves Labs), mouse anti-α7 (BIOMOL), mouse anti-α3 (BIOMOL), mouse anti-Rpt1 (BIOMOL), mouse anti-Rpt6 (mouse), mouse anti-Rpt3 (BIOMOL), rabbit anti-Rpt3 (BIOMOL), rabbit synapsinI (Chemicon). Appropriate HRP conjugated secondary antibodies (HRP-conjugated anti-mouse IgG ab, HRP-conjugated anti-rabbit IgG ab (Jackson ImmunoResearch Laboratories), and HRP-conjugated anti-chicken IgY ab (The Aves Labs)) were used for the detection with ECL Chemiluminescence Reagent (Amersham Biosciences).

Immunoprecipitation. In order to immunoprecipitate the endogenous proteasome, cultured hippocampal neurons expressing Rpt1-GFP were lysed in lysis buffer containing 1% NP-40. Immunoprecipitation was performed with a mouse monoclonal Rpt6 antibody (BIOMOL) and the immunoprecipitates were analyzed for an endogenous proteasome subunit Rpt3 (with a rabbit polyclonal antibody from BIOMOL) and Rpt1-GFP (with a rabbit polyclonal antibody from MOLECULAR PROBES).

Statistical Analysis. All of the error bars indicate the standard error of the mean. Non-paired two-way Student's t-test was used to compare the means of two treatments. For multiple groups, one-way ANOVA was performed (Fig. 1c, Fig. 3f,g). All of the data were tested for normal distribution by Anderson-Darling Test. GraphPad Prism v4.3 was used to compute the p values.

Supplementary Table 1:

Percent colocalization of Rpt1-GFP, endogenous proteasome subunits and various synaptic proteins.

Numbers indicate the percent of "protein A" puncta number that colocalizes with "protein B" puncta number.

Number that follows the ± sign is the standard error of the mean (s.e.m.).

PROTEIN B

PROTEINA	**Rpt1-GFP (total)**	**Bassoon**	**core**	**actinin**	**Rpt3**	**actin**	**Rpt1-GFP (in spines)**
Rpt1-GFP (total)			89.7 ±1.2*				
Bassoon							95.6 ± 1.2*
core	94.8 ±1.5*					39.5 ±1.0**	
actinin							95.6 ± 1.3***
Rpt3						42.9 ±1.6**	
actin			58.2 ± 1.5**		67.0±1.9**		
Rpt1-GFP (in spines)		97.8 ± 1.4*		97.1 ± 1.7***			

* Fig 2a,b (n=10 dendrites for both Rpt1-GFP/bassoon and Rpt1-GFP/core).

** Fig 5c (n=17 and 23 dendrites for Rpt3/actin and core/actin respectively).

*** data not shown (n=12 dendrites)

Supplementary Notes:

31. D'Apuzzo, M., Mandolesi, G., Reis, G. & Schuman, E. M. Abundant GFP expression and LTP in hippocampal acute slices by in vivo injection of sindbis virus. *J Neurophysiol* **86**, 1037-42 (2001).
32. Aakalu, G., Smith, W. B., Nguyen, N., Jiang, C. & Schuman, E. M. Dynamic visualization of local protein synthesis in hippocampal neurons. *Neuron* **30**, 489-502 (2001).
33. Smith, W. B., Starck, S. R., Roberts, R. W. & Schuman, E. M. Dopaminergic stimulation of local protein synthesis enhances surface expression of GluR1 and synaptic transmission in hippocampal neurons. *Neuron* **45**, 765-79 (2005).
34. Murase, S., Mosser, E. & Schuman, E. M. Depolarization drives beta-Catenin into neuronal spines promoting changes in synaptic structure and function. *Neuron* **35**, 91-105 (2002).
35. Bagni, C., Mannucci, L., Dotti, C. G. & Amaldi, F. Chemical stimulation of synaptosomes modulates alpha -Ca2+/calmodulin-dependent protein kinase II mRNA association to polysomes. *J Neurosci* **20**, RC76 (2000).